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Crypticity of *Myrothecium verrucaria* Spores to Maltose and Induction of Transport by Maltulose, a Common Maltose Contaminant

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Spores of the fungus *Myrothecium verrucaria* are cryptic to maltose and isomaltose. Induction of a transport system can be effected by several sugars whose order of effectiveness is: turanose > maltulose > sucrose > D-arabinose, D-fructose, nigerose, maltotriulose, kestose > melezitose, raffinose, nystose, and stachyose. The transport system is not specific to maltose and isomaltose, and it is apparently identical to an induced trehalose permease described previously. Induction of the permease is markedly influenced by spore age—older spores being more responsive. Pure maltose is not absorbed by spores. Absorption of commercial reagent-grade maltose is due to permease induction by maltulose as an impurity. Maltulose contamination of maltose was demonstrated by charcoal column chromatography and comparison of its physical, chemical, and permease-inductive properties with those of authentic maltulose. Maltose accumulates temporarily in spores after absorption and then decreases, although no conversion to glucose can be detected. Although spores contain small quantities of maltase, metabolism of maltose may be via some nonhydrolytic pathway.

While studying trehalose absorption in spores of the fungus *Myrothecium verrucaria* (11), it was found that two transport systems were involved—one constitutive and the other induced by exposure to turanose. On the other hand, certain other disaccharides—kojibiose, maltose, and isomaltose—were absorbed only after exposure of the spores to turanose, there being no constitutive transport system. Since this turanose treatment was shown to cause induction of a sugar transport system, it appeared that the spores were probably cryptic to these sugars. The phenomenon of "crypticity of certain cells toward certain substrates, i.e., their incapacity to metabolize a given substrate even though they possess the relevant enzyme systems" (1), has been observed in many microbial cells (5), but not, to our knowledge, in fungus spores.

The studies reported here provide evidence of the crypticity of fungus spores to certain α-D-glucosyl-D-glucoses and also demonstrate that commercial maltose preparations contain an impurity, maltulose, which is capable of inducing a maltose transport system, i.e., a permease.

MATERIALS AND METHODS

Spores of *M. verrucaria* QM 460 were harvested from agar cultures grown at 29 C with filter paper as carbon source and were washed before use, as described previously (7). Since sporulation is complete in about 5 days, spore age is equal to culture age minus 5 days. To obtain dry weights, spores were filtered on a fine sintered-glass funnel and dried at 100 C.

Sugar absorption was measured with spores suspended in 0.05 M KH_2PO_4-K_2HPO_4 buffer (*p*H 5.5) and incubated on a reciprocal shaker at 30 C. Sources of sugars are shown in Fig. 1 and Table 1. Maltose was also prepared from potato starch (Amend Drug and Chemical Co., New York, N.Y.) by treatment with β-amylase (Wallerstein Co., Staten Island, N.Y.) (14) followed by charcoal column chromatography of the enzyme digest.

Respiration was measured by standard Warburg techniques at 30 C. Reducing sugar was determined by the dinitrosalicylic acid method (20), total sugar by the orcinol reagent (17), and mannitol by the method of Frisell et al. (2).

Ketoses were determined spectrophotometrically by the method of Percheron (16).

Charcoal column chromatography of maltose samples was performed by the method of Whistler and Durso (24) with cocoanut charcoal (Fisher Scientific Co., Pittsburgh, Pa.; 50-200 mesh) which had been washed with 1 M hydrochloric acid and then with water until the effluent was neutral. The maltose used was from Matheson-Coleman Bell, East Rutherford, N.J. This preparation was absorbed in a manner

similar to maltose from Nutritional Biochemicals Corp. (curve 2, Fig. 1).

Chromatography on Whatman 3MM paper was effected by developing from 24 to 48 hr in ethyl acetate-acetic acid-water (9:2:2, v/v) (13); carbohydrates were generally located with silver nitrate-sodium hydroxide reagents (23), reducing sugars with aniline oxalate (3), and ketoses with orcinol (6).

TABLE 1. *Activity of various sugars as inducers of the maltose transport system*

Sugar	Configuration[a]	Activity[b]	Source[c]
D-Arabinose		53	8
L-Arabinose		5	8
D-Ribose		5	10
D-Xylose		5	6
2-Deoxy-D-glucose		0	8
D-Fructose		55	8
D-Galactose		(0)	11
D-Glucose		5	5
D-Glucosamine		(0)	8
Methyl-α-D-glucopyranoside		0	8
Methyl-β-D-glucopyranoside		0	8
α,α-Trehalose	G(α 1-1)G	0	7
Kojibiose	G(α 1-2)G	0	1
Nigerose	G(α 1-3)G	50	3
Maltose	G(α 1-4)G	0	—
Isomaltose	G(α 1-6)G	0	2
Sucrose	G(α 1-2)F	110	6
Turanose	G(α 1-3)F	170*	7
Maltulose	G(α 1-4)F	140	2
Palatinose	G(α 1-6)F	0	2
Melibiose	Gal(α 1-6)G	(0)	8
Melibiulose	Gal(α 1-6)F	6	2
β,β-Trehalose	G(β 1-1)G	(0)	2
Sophorose	G(β 1-2)G	(0)	3
Laminaribiose	G(β 1-3)G	(0)	3
Cellobiose	G(β 1-4)G	0	8
Gentiobiose	G(β 1-6)G	(0)	9
Gentiobiulose	G(β 1-6)F	5	2
Lactose	Gal(β 1-4)G	(0)	4
Rutinose	deoxy M(β 1-6)G	6	3
Rutinulose	deoxy M(β 1-6)F	6	2
Nigerotriose	G(α 1-3) G (α 1-3)G	0	3
Maltotriose	G(α 1-4) G (α 1-4)G	0	2
Melezitose	G(α 1-2) F (α 1-3)G	20	8
Maltotriulose	G(α 1-4) G (α 1-4)F	55	2
Raffinose	Gal(α 1-6) G (α 1-2)F	20	7
Kestose	G(α 1-2) F (β 1-2)F	45	12
Nystose	G(α 1-2) F (β 1-2) F (β 1-2)F	20	12
Nigerotetraose	G(α 1-3) G (α 1-3) G (α 1-3)G	0	3
Tetramer from nigeran	G(α 1-3) G (α 1-4) G (α 1-3)G	8	3
Stachyose	Gal(α 1-6) Gal (α 1-6) G (α 1-3)F	15	7

[a] G = glucose; Gal = galactose; F = fructose; deoxy M = deoxymannose.

[b] Rate of absorption of maltose in μg per mg of spores per hr. Inducers at 100 μg/ml except turanose (*) which was at 10 μg/ml. Numbers in parentheses indicate tested only with trehalose as substrate.

[c] (1) K. Aso; (2) F. W. Parrish; (3) E. T. Reese; (4) Baker and Adams; (5) Eastern Chemical Corp., Pequannock, N.J.; (6) Fisher Scientific Co., Pittsburgh, Pa. (chemically pure); (7) Nutritional Biochemicals Corp., Cleveland, Ohio; (8) Pfanstiehl Chemical Corp., Waukegan, Ill. (chemically pure); (9) Pillsbury Chemicals, Detroit, Mich.; (10) Schwarz Laboratories, Inc., Mt. Vernon, N.Y.; (11) Sigma Chemical Co., St. Louis, Mo.; (12) W. Binkley.

Ion-exchange chromatography was performed on Amberlite IR-120 (H^+) and Duolite A-4 (HCO_3^-) resins.

Trimethylsilyl ethers of maltose samples were prepared as described by Sweeley et al. (22), but with the use of *N,N*-dimethylformamide instead of pyridine, and were fractionated on silica gel (Merck, distributed by Brinckmann Instruments, Inc., Great Neck, N.Y.; 0.05 to 0.20 mm) with etherhexane (1:15). Deetherification was effected with boiling 50% methanol to regenerate the parent reducing sugar.

β-Maltose octaacetate was prepared by acetylating maltose with acetic anhydride and anhydrous sodium acetate and crystallizing from ethyl alcohol; the acetate had a melting point of 159 C and a specific rotation of +62.6° (5% solution in chloroform), in agreement with previously recorded values (4). Deacetylation of the octaacetate with sodium methoxide in methanol and crystallization from aqueous ethyl alcohol gave β-maltose monohydrate having a melting point of 103 C and specific rotation of +130° (2% aqueous solution).

RESULTS

Effect of source of maltose. Whereas some sugars, D-glucose, for example, are absorbed by *M. verrucaria* spores with no lag, absorption of commercial, reagent-grade maltose occurred only after a lag of variable duration, depending on the source of the maltose (Fig. 1). The rate of absorption was also affected markedly—the shorter the lag, the more rapid the absorption. Furthermore, the rate of absorption decreased after passing a maximum, and complete absorption occurred only after prolonged incubation. Since previous data (11) had shown that exposure of spores to traces of turanose could induce them to absorb maltose, it was hypothesized that some impurity in the maltose preparations was responsible for the observed differences.

Maltose prepared by hydrolysis of potato starch by β-amylase was absorbed slowly, at a rate similar to that of Difco Certified material.

Attempts to remove inducer from commercial maltose samples by chromatography of the trimethylsilyl ether derivative were not successful.

Increased absorption occurred when maltose samples were treated with weak-base ion-exchange resin or when maltose prepared from octaacetate was used (*see* Discussion).

Fractionation of maltose. Paper chromatography of maltose samples showed the presence of traces of D-glucose, maltotriose, and ketoses, but not at concentrations that could be correlated with rate of maltose absorption. However, inducer was mainly in that portion of the maltose region nearer the solvent front.

Charcoal column chromatography permitted isolation of a disaccharide impurity, eluted just before maltose, containing D-glucose and D-fructose. This disaccharide had identical physical properties with authentic maltulose, and both sugars gave the same osazone derivative (15) and the same spectrophotometric response with thiobarbituric acid reagent. Comparison with authentic maltulose as an inducer of the maltose transport system showed comparable response (Fig. 2). While maltulose was not isolated from all samples of maltose, its presence could be demonstrated by paper chromatography.

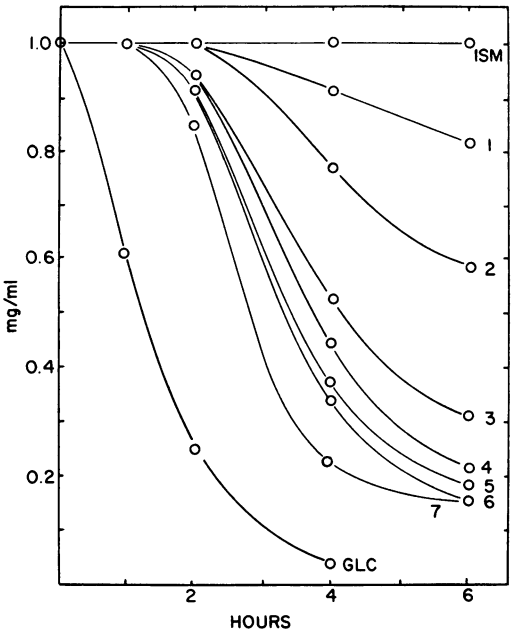

FIG. 1. *Absorption of different maltose samples, of isomaltose (ISM) and of D-glucose (GLC). Spores 21 days old, 8.95 mg/ml; substrates at 1 mg/ml. 1 = Difco Certified; 2 = Nutritional Biochemicals; 3 = Eastman; 4 = Pfanstiehl (24630); 5 = Calbiochem; 6 = Fisher; 7 = Pfanstiehl (7304N).*

Effect of spore age. Spore age has a marked effect on sugar absorption (11) and also on the response of spores to inducers of sugar transport systems (Fig. 3). Spores 40 days old absorbed D-glucose about twice as fast as those 17 days old. Maltose was absorbed by young turanose-treated spores only slightly faster than D-glucose. Maltose absorption by untreated older spores is ascribed to greater induction by the maltose impurities. Thus, isomaltose was not absorbed by untreated older spores.

Induction of the maltose transport system. Induction of the maltose transport system was dependent, among other things, upon the nature and concentration of inducer and the time of preincubation with inducer prior to addition of

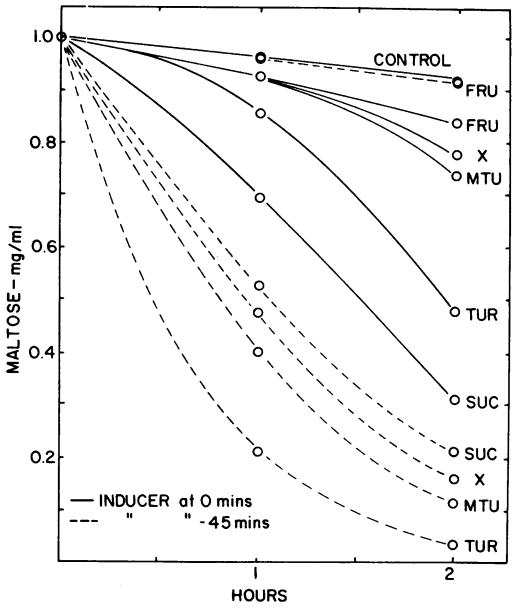

FIG. 2. *Comparison of maltose impurity with other sugars as inducers of maltose transport. Spores 24 days old at 5.75 mg/ml; inducers at 10 μg/ml added simultaneously with maltose (solid lines) or 45 min prior to maltose (dashed lines). (Note: sucrose at 100 μg/ml for the 45-min pretreatment data.) FRU = fructose; MTU = maltulose; TUR = turanose; SUC = sucrose; X = maltose impurity; C = control, no inducer.*

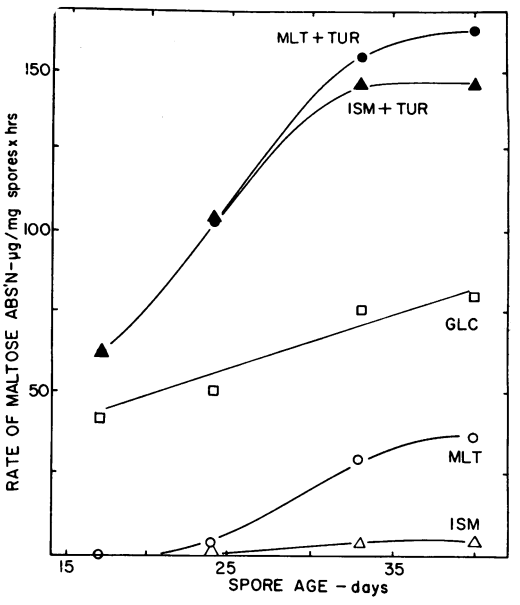

FIG. 3. *Effect of spore age on absorption of glucose (GLC) and of maltose (MLT) and isomaltose (ISM) by control and turanose-treated spores. Turanose (TUR) at 10 μg/ml added 45 min before substrates at 1 mg/ml.*

substrate. The effects of spore age are pronounced and have been discussed in the preceding section. Quantitation of the relative effectiveness of various inducers is complicated by these multiple factors.

The activity of the transport system, assuming it is proportional to rate of maltose absorption, increased up to a maximum with increased time of preincubation with inducer. Continued preincubation resulted in a decreased rate of substrate absorption (Fig. 4). The time interval between addition of inducer and attainment of the maximal substrate transport rate varied considerably with different inducers. With D-arabinose, D-fructose, or sucrose as inducer, attainment of a maximal rate of maltose absorption occurred quickly, so that preincubation with inducer prior to addition of substrate had little effect. On the other hand, with turanose or maltulose as inducer, preincubation for periods in excess of 30 min was necessary for maximal induction.

In the above experiments, the inducer was not removed from the medium prior to addition of substrate. Similar results were obtained in other experiments in which the inducer (turanose) was

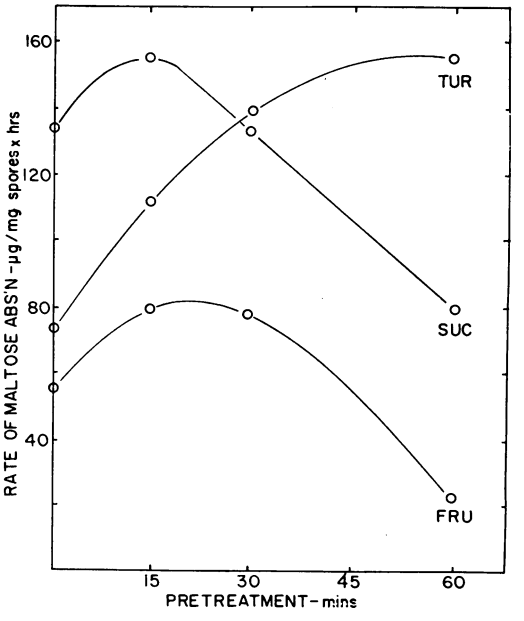

FIG. 4. *Effect of time of preincubation with inducer on development of maltose transport system. Spores 26 days old at 5.2 mg/ml; maltose at 1 mg/ml; inducers at 100 μg/ml. TUR = turanose; SUC = sucrose; FRU = fructose.*

removed from the spores by centrifugation and washing prior to addition of substrate (Fig. 5). It should be noted that reversal of the curve was not due to depletion of the turanose inducer.

A number of sugars were screened for activity as inducers (Table 1), and the more active of these were tested at various concentrations (Fig. 6). Turanose was by far the most effective inducer, followed by maltulose and sucrose. D-Arabinose, D-fructose, nigerose, maltotriulose, and kestose had comparable moderate activity. Melezitose, raffinose, nystose, and stachyose were slightly active. The activity of sugars such as L-arabinose is of questionable significance.

Maltose metabolism: comparison with glucose and threhalose. Respiratory activity, as well as changes in total soluble carbohydrate, mannitol, and reducing sugars in spore extracts, determined during absorption of maltose, D-glucose, and trehalose by turanose-treated spores showed distinctly different patterns (Fig. 7). With maltose as substrate, accumulation occurred within the spores for the first hour and then declined. (Paper chromatography showed the reducing sugar to be maltose.) Mannitol also increased. With D-glucose as substrate, no increase in reducing sugars was detected, but mannitol content increased. With trehalose as substrate, the changes in mannitol were insignificant, whereas considerable trehalose accumulated (*see also* 11). Thus, D-glucose is metabolized as fast as it enters the cell, whereas maltose enters faster than it is metabolized initially, but then is metabolized faster than it accumulates. Initial composition of the spores, in μg/mg of spore dry weight, was: trehalose, 155; mannitol, 14.

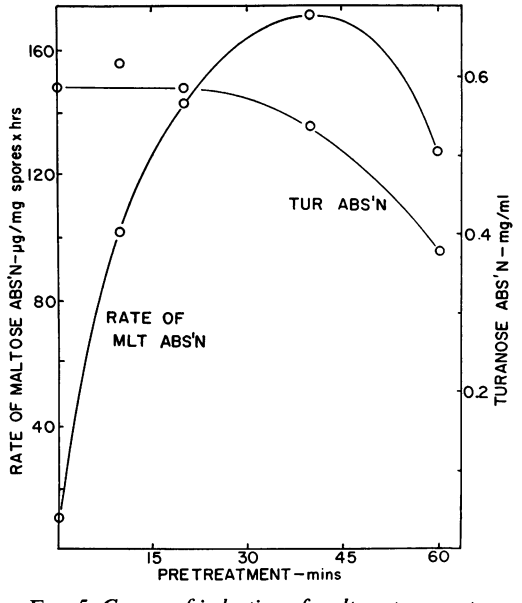

FIG. 5. *Course of induction of maltose transport system by turanose and absorption of turanose during induction. Spores 20 days old at 12 mg/ml. Turanose at 1 mg/ml. Spores washed twice to remove turanose prior to addition of maltose at 1 mg/ml.*

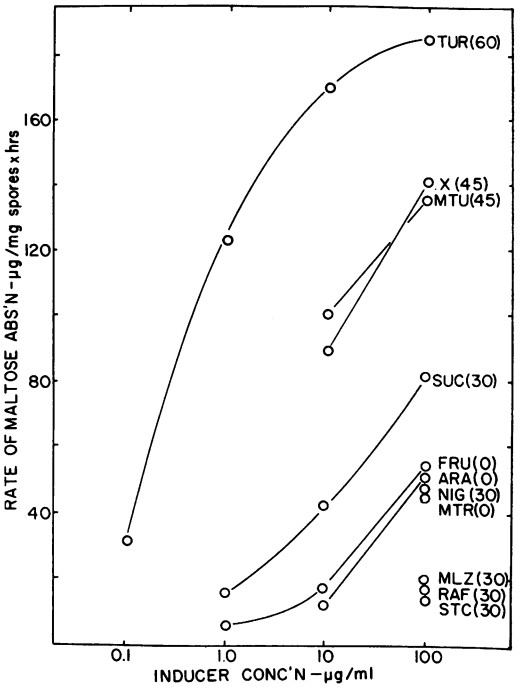

FIG. 6. *Effect of concentration of inducers on absorption of maltose (1 mg/ml). ARA = D-arabinose; FRU = D-fructose; MTU = maltulose; NIG = nigerose; SUC = sucrose; TUR = turanose; MLZ = melezitose; RAF = raffinose; STC = stachyose; X = maltose impurity; MTR = maltotriulose. Numbers in parentheses indicate duration of pretreatment in minutes.*

Transport of maltose and trehalose was against a concentration gradient. Assuming the volume of suspended spores to be 3.1 μliters/mg of dry weight (11), the internal maltose concentration was calculated to be 53 mM at 1 hr, and of trehalose to be 222 mM. Initial external concentration of substrates was 5.6 mM and of internal trehalose was 146 mM.

DISCUSSION

M. verrucaria spores absorb maltose from solutions of commercial preparations after a lag period, whereas purified maltose is not absorbed. This absorption of maltose from reagent grade samples has been shown to be due to induction of a transport system by maltulose, a contaminant present at trace levels (up to 0.2%) in all

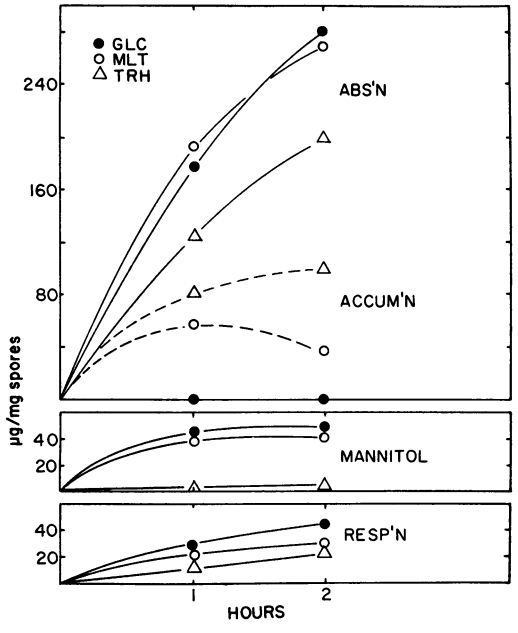

FIG. 7. *Metabolism of* D-*glucose (GLC), maltose (MLT), and trehalose (TRH): absorption, accumulation, respiration, and changes in mannitol content of spores. Spores 33 days old at 4.4 mg/ml pretreated with turanose at 10 μg/ml for 45 min, centrifuged, and washed prior to adding substrates at 2 mg/ml. Respiration data calculated in terms of μg of sugar oxidized/mg of spores, assuming complete oxidation to* CO_2 *and water and corrected for endogenous respiration.*

samples tested. The origin of this maltulose (α-D-glucopyranoside (1–4)-D-fructose) is not known, but it is presumed to arise from alkaline isomerization of maltose, possibly by contact with basic ion-exchange resin in processing. An increased rate of absorption of maltose was observed when Difco Certified maltose was passed through Duolite A-4 weak-base resin at room temperature. We did not find evidence for the presence of maltulose in the β-amylase digest of potato starch; we assume that maltose is obtained commercially by this procedure. Similarly, the increased rate of absorption of maltose produced by deacetylation of β-maltose octaacetate is probably due to the formation of some maltulose; Suzuki and Hehre (21) have reported the formation of traces of D-fructose during deacetylation of D-glucose pentaacetate with sodium methoxide.

We were unable to render maltose free from inducer by chromatography of the trimethylsilyl ether derivative; this approach was attempted because of the mild conditions required for the formation of the ether and its subsequent hydrolysis to the parent sugar. When the inducer had been characterized as maltulose, we showed that the trimethylsilyl ethers of maltose and maltulose are not separable under our conditions.

This demonstration of physiological activity by trace carbohydrate contaminants in presumably pure simple sugars is not unique. Other studies have shown, for example, that: induction of cellulase in *Trichoderma viride* by glucose is due to traces (0.006%) of sophorose contamination (12); glucose contamination of maltose decreases its capacity for induction of α-glucosidase in *Bacillus cereus* (19); an oligosaccharide containing 2,5 anhydro-L-idose induces respiratory enzymes in yeast (18).

The transport system induced by maltulose is apparently identical with the turanose-induced trehalose transport system (11). Evidence indicates that this induced permease effects the transport of the α-glucosyl-glucose linked disaccharides trehalose (α 1–1), maltose (α 1–4), and isomaltose (α 1–6). Since the turanose-induced system was shown also to effect the transport of kohibiose, nigerose, turanose, and melezitose, it is assumed that the maltulose-induced system will also cause transport of these sugars. Absorption of β-glucosyl-glucose linked disaccharides is apparently effected by a constitutive transport system(s).

Further studies of the ability of other sugars to induce this transport system show that the specificity of inducers is not as limited as was throught previously (11). Thus, a number of sugars in addition to turanose and nigerose are effective (*see* Table 1 and accompanying comments).

The activity of sucrose as an inducer is probably not due to its hydrolysis to fructose by the surface-located invertase (8), since induction by sucrose occurs at much lower concentrations than by fructose. Equimolar concentrations of D-glucose and D-fructose have the same activity as D-fructose alone. Since the invertase of these spores is a fructosidase (9), it is unlikely that activity of melezitose as an inducer is due to enzymatic hydrolysis, although the activity of raffinose could be so ascribed.

In attempting to correlate structure of inducers with activity, it is noteworthy that all active sugars tested contain fructose, although not all sugars containing fructose were active. The only exception to this is nigerose. Structurally, D-arabinose is closely related to D-fructose, i.e., the configuration of D-arabinose, about C-2 to C-5, is identical to that of D-fructose, about C-3 to C-6. Available evidence indicates that the same transport system is activated by all of these inducers.

The manner in which maltose is metabolized is not known. The spores do contain maltase, as can be demonstrated by incubating toluene-treated spores with maltose, under which conditions the rate of hydrolysis is about 15 μg of glucose produced per mg of spores per hour. This level is approximately adequate to account for respiratory activity on maltose. If metabolism of maltose requires the mediation of maltase, then maltase activity must be the factor limiting maltose metabolism once penetration has occurred, since maltose accumulates in the cells and no glucose is found. In view of other studies on the metabolism of trehalose and sucrose by these spores (9, 10), the involvement of nonhydrolytic pathways, such as by a phosphorolyase of transglycosidase, must be considered.

M. verrucaria spores are cryptic to isomaltose as well as to maltose. Transport of isomaltose is effected by the same induced permease that causes maltose transport.

It is interesting to compare the events following absorption of D-glucose, maltose, and trehalose. Glucose appears to be metabolized directly as fast as it is absorbed. Both maltose and trehalose accumulate in the cells at concentrations in excess of that in the external medium. Whereas maltose decreases after reaching a maximum, trehalose that is not metabolized immediately goes into the "permanent" endogenous trehalose pool. Mannitol content of the spores increases following absorption of glucose or maltose, but not of trehalose.

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